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<b>13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)</b> We recently identified a novel transcription factor, LMO-4, which exhibits prominent expression in epithelial cells, including that of the breast. Since previous members of the LIM only (LMO) gene family are oncogenes in lymphocytes, we hypothesized that LMO-4 may play a role in mammary gland development and cancer. We have now shown that expression of LMO-4 is associated with undifferentiated cellular stage of breast epithelial cells, such as that found during lobuloalveolar development in pregnancy and in breast cancer. We have created transgenic mice in which we overexpress a dominant negative LMO-4 under the MMTV promoter (MMTV-engrailed-LMO4) to test the role of LMO-4 in mammary gland development. Our results show significant inhibition of lobuloalveolar development in these MMTV-engrailed-LMO4 transgenic mice, indicating that LMO-4 plays roles in proliferation and/or invasion of breast epithelial cells. Because these cellular features are associated with breast carcinogenesis and because LMO-4 is overexpressed in a subset of breast cancers, our studies implicate LMO4 as a possible oncogene in breast cancer. We have also created stable breast cancer cell lines in which we can induce expression of LMO4 and Clim2, thus allowing us in future work to identify interacting proteins and study the role of these genes on breast cancer cell proliferation.							
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### **A. Abstract**

## **INTRODUCTION:**

LIM only factors, LMOs, are nuclear proteins composed of two LIM domains and little other sequence. Two of the founding members of this family are act as oncogenes in lymphocytes. We have recently identified a new member of this family, LMO-4, which is highly expressed in proliferating epithelial cells, including those of the breast. In addition, LMO-4 has been shown to be highly expressed in ductal breast carcinoma. Two mechanisms of action have been proposed for LMOs. First, they may bind the LIM homeodomain co-activator CLIM thereby interfering with the activity of LIM homeodomain proteins. Second, LMOs may associate with DNA-binding proteins, thus attracting the coactivator CLIM to DNA. Our hypothesis is that LMO-4 plays a role in regulation of breast epithelial cell proliferation and that subversion of its function may play a role in breast cancer. Further, that LMO-4 acts, at least in part, by associating with DNA-binding proteins.

Our specific aims are: #1. To test the potential role of the LMO-4/CLIM complex in breast development and breast cancer by using the MMTV promoter to overexpress LMO-4 and a dominant negative form of CLIM in breast epithelial cells. #2. We have recently identified a KRAB Zinc finger transcription factor, Znf43, as a potential interacting partner of LMO-4. We propose to test the relevance of this interaction using human breast carcinoma cell lines. We will test the effect on proliferation and tumor growth.

## **BODY:**

### ***Task 1. Test the effect of overexpressing LMO-4 and a dominant negative CLIM in the breast of transgenic mice.***

#### **1. Create and characterize transgenic constructs.**

The initial plan was to create two lines of transgenic mice: MMTV-LMO4 and MMTV-dominant negative-Clim. In addition, I have worked on a third transgenic line where we have created a dominant negative LMO4 molecule by linking LMO4 to the Engrailed repression domain. This construct is referred to as MMTV-Engrailed-LMO4. All three transgenic constructs have been created and characterized.

#### **2. Injecting transgenic constructs into oocytes.**

All three constructs, MMTV-LMO4, MMTV-dominant negative-Clim and MMTV-Engrailed-LMO4, have been injected into oocytes for generation of transgenic mice.

#### **3. Analyses of founder mice.**

Founder mice have been generated for all three constructs. The work with MMTV-dominant negative-Clim and MMTV-Engrailed-LMO4 mice is farther along than the work with the MMTV-LMO4 mice. For the MMTV-dominant negative-Clim construct, we obtained 4 transgenic founder mice out of a total of 57 pups. Out of these 4 transgenic founders, 2 expressed the transgene and were selected for further analyses. For the MMTV-Engrailed-LMO4 construct, we obtained 5 transgenic mice out of 13 pups.

Out of these 5 transgenic founders, three were found to express the transgene and were selected for further analyses. We recently obtained 7 positive founder mice out of 19 pups for the MMTV-LMO4 construct (Fig. 1). The positive mice can now be bred with wild-type mice to determine which founders are expressing.

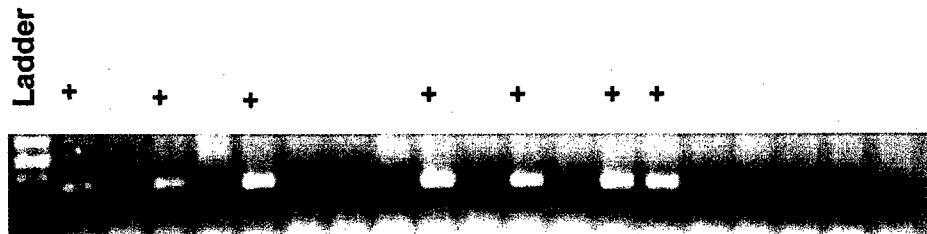


Figure 1. PCR genotyping of MMTV-LMO4 founder mice. Positive mice are indicated with +.

#### 4. Generating transgenic progeny.

Transgenic progeny have been generated for the MMTV-dominant negative-Clim and MMTV-Engrailed-LMO4 mice. No progeny have been generated for the MMTV-LMO4 mice.

#### 5. Analyses of transgenic mice.

To focus our study on the possible role of LMO4 and Clim2, we have outlined the expression of LMO-4 during mammary gland development and correlated it with expression of the associated co-factor, Clim2. In these studies (Fig. 2), mRNA expression was analyzed by RNAse protection assays, using  $^{32}\text{P}$ -labeled antisense riboprobes. Yeast tRNA was used as a negative control, and actin as internal control for variations in RNA quantity and quality. The results show that LMO4 and Clim2 transcript levels are coordinately and greatly upregulated during mid-pregnancy, a stage in mammary gland development when epithelial cells are undergoing proliferation and invasion into the fat pad. Another expression peak is observed during lactation suggesting possible additional roles during this stage. This expression pattern suggests the possibility that the LMO-4/Clim2 complex plays roles in maintaining proliferation and/or suppressing differentiation – two features that characterize breast cancer cells.

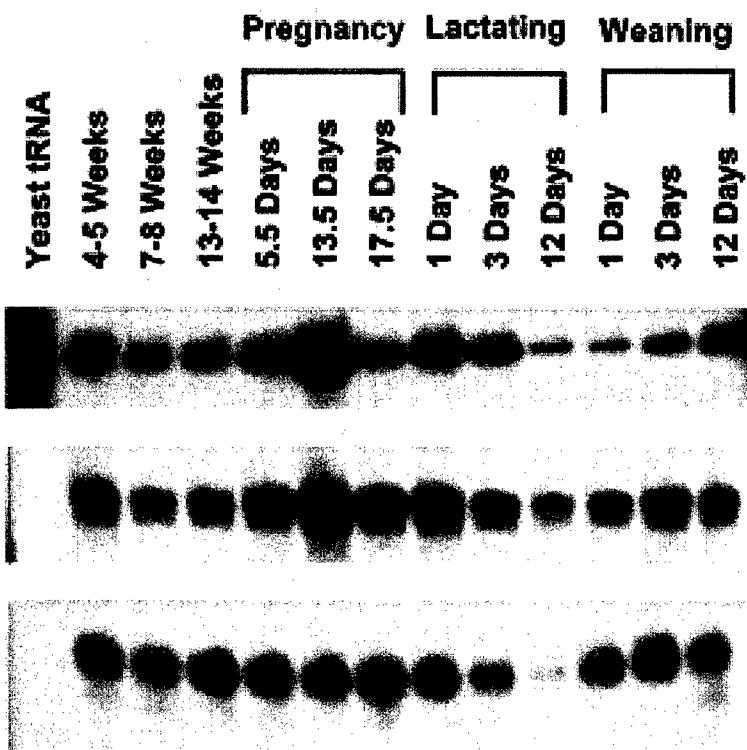
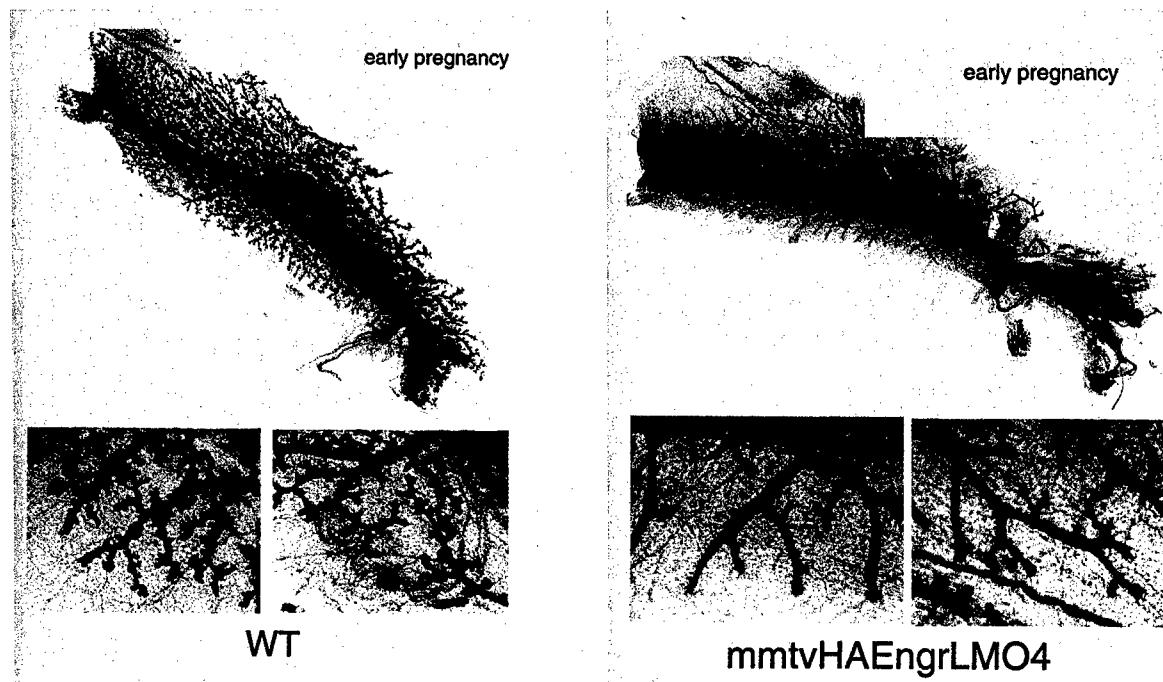


Figure 2. Expression of LMO4 (top panel), Clim2 (middle panel) and actin (bottom panel) transcripts during mammary gland development.

Analysis of the transgenic mice is progressing but these experiments show that the MMTV-Engrailed-LMO4 mice exhibit decreased lobuloalveolar development both during pubertal development and during early and mid-pregnancy (Fig. 3). These results are consistent with our hypothesis derived from the expression analyses and indicate that LMO4 is likely to play roles to promote invasion and/or proliferation of mammary gland epithelial cells. We have not observed a clear phenotype in the MMTV-dominant negative-Clim lines.



### Figure 3.

The figure shows whole mount analyses of mammary glands at day 5.5 of pregnancy, comparing wild-type mice to MMTV-*engrailed-LMO4* mice. Clear inhibition of lobuloalveolar development is observed in the transgenic mice. Similar findings were observed during mid-pregnancy (data not shown).

## Task 2. Test the relevance of LMO-4/Znf43 interactions in breast cancer cell lines.

### 1. Creating constructs and cell lines transfections.

We have initiated characterization of LMO-4 interacting factors by creating LMO-4 and Clim-2 expressing vectors that can be induced by tetracyclin. The LMO-4 protein is tagged with Myc and the Clim-2 protein is tagged with HA, thus allowing specific immunoprecipitation of these proteins from breast cancer cell lines. The construction of both vectors is completed.

### 2. Performing in vitro interaction assays in vitro and in cells.

These studies await further characterization and expansion of the stable MCF-7 cancer cell lines.

### 3. Creating stable cell lines for analyses.

The vectors described above have been stably introduced into MCF-7 breast cancer cell lines. This work has progressed well and we have already isolated several MCF-7 cell lines in which we can induce expression of LMO4 and Clim2 (Fig. 4). After expansion of these cell lines we can now proceed with immunoprecipitation studies to characterize interacting factors and test the effect of LMO-4 and Clim2 on cell growth.

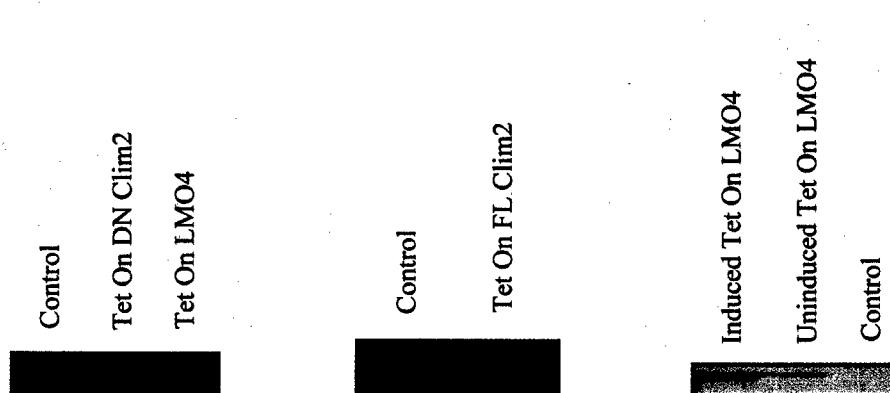


Figure 4. The left panel shows western blot detecting expression of DN-Clim and LMO-4 in pools of MCF-7 cells in response to tetracyclin. The middle panel shows tet-inducible expression of full length Clim2 under same conditions. The panel to the right shows results from a isolated MCF-7 cell clone where LMO-4 expression is induced by tet treatment for 48 hours.

## **KEY RESEARCH ACCOMPLISHMENTS DURING LAST YEAR:**

1. Definition of LMO4 and Clim2 gene expression during mammary gland development.
2. Obtaining founder transgenic mice positive for MMTV-LMO4.
3. Establishment of transgenic lines and beginning analyses for MMTV-Engrailed-LMO4 and MMTV-dominant negative-Clim.
4. Showing that MMTV-Engrailed-LMO4 mice exhibit defective lobuloalveolar development, consistent with an important role for LMO4 in promoting proliferation and/or invasion of breast epithelial cells.
5. Creation of stable breast cancer cell lines expressing tagged LMO4 and Clim2 proteins, suitable for testing protein-protein interactions with immunoprecipitations, and for testing the effect of LMO4/Clim2 on cancer cell proliferation.

## **REPORTABLE OUTCOMES TO DATE:**

1. Transgenic mouse models for LMO expression
2. Permanent breast cancer cell lines expressing tagged LMO4 and Clim2
3. Manuscript in preparation: Wang, N., Kudryavtseva, E., Chen, I., Sugihara, T.M., McCormick, J., and Andersen, B. 2002. LMO-4 plays a role in lobuloalveolar development in the mammary gland. *In preparation*.
4. Abstract: Wang, N., Kudryavtseva, E., Chen, I., Sugihara, T., & Andersen, B. 2002. The potential role of a new LIM factor, LMO4, in breast cancer. Proceedings Era of Hope Meeting, Orlando Florida, September (Abstract P4-1).

## **CONCLUSIONS:**

In summary, I have made significant progress on both specific aims, and my training in breast cancer biology has been greatly enhanced. Our results show that LMO-4 expression is associated with undifferentiated breast epithelial cells such as those found during mid-pregnancy and in breast cancer. The major achievement during the last year is the finding that interfering with LMO4 in breast epithelial cells leads to inhibition of lobuloalveolar development in mice. This finding, which we reported during the last Era of Hope meeting, strengthens our hypothesis that overexpression of LMO4 may contribute to breast carcinogenesis. With our work, we hope to generate new ideas about treatment of breast cancer, thus impacting on reducing the human/economic cost of breast cancer

## **THE POTENTIAL ROLE OF A NEW LIM FACTOR, LMO4, IN BREAST CANCER**

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Many properties of breast cancer cells, including increased proliferation and invasion, are common to epithelial cells of the developing mammary gland, suggesting that understanding of developmental control in normal mammary glands may provide important insights into the biology of breast cancer. This notion is supported by work in many organ systems, demonstrating that subversion of developmental control genes plays roles in carcinogenesis. LIM domain factors and associated co-regulators are important developmental regulators involved in pattern formation and organogenesis in a wide spectrum of organisms, including mammals. We isolated a LIM only factor, LMO-4, which is highly expressed in epithelial cells, including mammary epithelium. Interestingly, LMO factors are known to be oncogenic in lymphocytes where their overexpression causes acute lymphocytic leukemia.

We have studied expression of LMO-4 in mammary glands of mice and found that it is most highly expressed in proliferating mammary epithelial cells during pregnancy, suggesting that the LMO-4 gene may play a role in proliferation. Since LMOs do not bind to DNA it is likely that they regulate transcription by interacting with DNA-binding proteins and transcriptional co-regulators. To search for such factors, we have screened a human breast cDNA library with LMO-4 as bait in the yeast two hybrid system and found several potential interacting partners, including DNA-binding proteins, Clim/Nli/Ldb co-regulators and a splicing factor previously shown to be amplified in breast cancer cell lines. To test the role of LMO-4 in mammary gland biology, we have generated three lines of transgenic mice expressing under control of the MMTV promoter a) wild-type LMO-4, b) LMO-4 fused to the VP-16 transactivation domain and c) LMO-4 fused to the engrailed repression domain. Whole mount mammary gland analyses of these transgenic mice is in progress and preliminary results will be presented. Analyses of the EST databases indicate that LMO-4 is highly expressed in mammary carcinomas and we are in the process of evaluating its expression in breast cancer.

We conclude that LMO-4 may be an important regulator of mammary epithelial cells and propose a hypothesis that its high level expression in mammary tumors may play a role in mammary carcinogenesis.